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The β-catenin/Tcf4/survivin signaling maintains a less differentiated phenotype and high proliferative capacity of human corneal epithelial progenitor cells

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ABSTRACT

It is clear that the microenvironment or niche plays an important role in determining the fate of stem cells: being stem cells or differentiated. However, the intrinsic pathways controlling the fate of adult stem cells in different niches are largely unknown. This study was to explore the role of β-catenin/Tcf4/survivin signaling in determining the fate of human corneal epithelial stem cells in different media. We observed that the low calcium serum-free media, especially CnT-20, promoted proliferative capacity, colony forming efficiency and stem cell-like phenotype of human corneal epithelial cells (HCECs) when compared with the cells cultured in a high calcium serum-containing medium SHEM. Three key factors in Wnt signaling, β-catenin, Tcf4 and survivin, were found to be expressed higher by HCECs grown in CnT-20 than those cultured in SHEM, as evaluated by real-time PCR, Western blotting and immunostaining. Transfection of siRNA-Tcf4 at 10–50 nM knocked down Tcf4, and also significantly suppressed its down stream molecule survivin at both mRNA and protein levels in HCECs. Furthermore, Tcf4 silencing significantly suppressed the proliferative capacity of HCECs, measured by WST-1 assay, compared with the control groups, untreated or transfected with non-coding sequence siRNA-fluorescein. These findings demonstrate that low calcium serum free media promote ex vivo expansion of corneal epithelial progenitor cells that retain a less differentiated phenotype and high proliferative capacity via β-catenin/Tcf4/survivin signaling, a novel intrinsic pathway. This study may have high impact and clinic implication on the expansion of corneal epithelial stem cells in regenerative medicine, especially for ocular surface reconstruction.

1. Introduction

The ocular surface is an ideal region to study epithelial stem cell biology because of the unique spatial arrangement of stem cells and transient amplifying cells (Tseng, 1989; Dua and Azuara-Blanco, 2000; Lavker et al., 1991; Diaz-Flores et al., 2006). The corneal epithelial stem cells have been identified to reside in the basal layer of limbal epithelium over last two decades. Limbal epithelial stem cells exhibit unique characteristics that satisfy the widely accepted criteria for defining adult stem cells, including (1) slow cycling or long cell cycle time during homeostasis *in vivo*; (2) small size and poor differentiation with primitive cytoplasm; (3) high proliferative potential after wounding or placement in culture; (4) ability for self-renewal and functional tissue regeneration (see review articles by Sun and Lavker, 2004; Watt and Hogan, 2000; Lavker and Sun, 2000; Cotsarelis et al., 1999). Both intrinsic and extrinsic signals regulate stem cell fate including adult stem cells. Through interaction with intrinsic signals, the extrinsic niche or the stem cell microenvironment is believed to be important in maintaining the "stemness" of the stem cells, including corneal epithelial stem cells (German et al., 2006; Schlotzer-Schrehardt and Kruse, 2005; Notara et al., 2010; Li et al., 2008). For example, it is well known that low calcium, serum-free culture media can provide an ideal niche in vitro to maintain or promote progenitor cell properties, such as proliferative capacity and undifferentiation status (Boyce and Ham, 1983; Kruse and Tseng, 1991; Litvinov et al., 2006; Loo et al., 2008), while high calcium and serum-containing media promote cell differentiation (Bertolero et al., 1986; Vicanova et al., 1998; Kawakita et al., 2004). However, the underlining molecular mechanisms by which the niche determines the stem cell fate are far from being completely elucidated.

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Wnt signaling pathway has been recognized to control a variety of functions and properties in various types of stem cells. Wnt signaling can be activated by niche factors to maintain stem cells in a self-renewing state (Rattis et al., 2004; Nemeth and Bodine, 2007; Fleming et al., 2008). During tissue development and regeneration, Wnt signals ensure the proper balance between proliferation and differentiation (Gat et al., 1998; Lo et al., 2004; Zhang et al., 2008). Wnt proteins are active in a variety of stem cells, including embryonic, hematopoietic, neural and mammary stem cells, as well as corneal epithelial stem cells (Rattis et al., 2004; Willert et al., 2003; Kawakita et al., 2005). The hallmark of the Wnt signaling pathway is the accumulation of the junctional protein β-catenin in the cytoplasm, which then translocates to the nucleus to trigger the β-catenin/Tcf enhancer factor transcriptional machinery, and upregulate target genes, such as survivin and c-myc (Graham et al., 2001; Poy et al., 2001; Wei et al., 2010). A classic example of the importance of this pathway is in the digestive tract, where in the crypt of the colon the loss of transcription factor T cell factor 4 (Tcf4), a key factor of canonical Wnt signaling pathway, leads to depletion of stem cells (Wei et al., 2010; Korinek et al., 1998). After activation by β-catenin/Tcf4 complex, survivin enhances cell proliferation while protecting cells from apoptosis (Kim et al., 2003; Zhu et al., 2010). Recently, Tcf4 and Tcf3 have been found to play a vital role in long-term maintenance and wound repair of both epidermis and hair follicles (Nguyen et al., 2009). However, the role of the Wnt pathway, particularly, β-catenin/Tcf4/survivin signaling in maintaining the properties of adult stem cells has not been elucidated. The purpose of present study was to explore the important role of Tcf4 signaling in determining the fate of corneal epithelial stem cells, using an in vitro culture model with different media providing niche factors: low calcium and serum free versus high calcium and serum containing.

2. Materials and methods

2.1. Materials and reagents

Cell culture dishes, plates, centrifuge tubes, and other plastic ware were purchased from Becton Dickinson and Company (Franklin Lakes, NJ). Nunc Lab-Tec II eight-chamber slides were from Nalge Nunc International Corp. (Naperville, IL). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). CnT-20 and CnT-50 progenitor media were from Chemicon International (Temecula, CA). Dulbecco modified Eagle's medium (DMEM), Ham F-12, Keratinocyte-SFM (KSFM) and Defined KSFM (D-KSFM), amphotericin B, gentamicin, 0.25% trypsin/EDTA solution, mouse monoclonal antibody (mAb) against connexin 43 (Cx43), and fluorescein Alexa-Fluor 488 conjugated secondary antibodies (Donkey anti-Goat IgG, Goat anti-rabbit or Goat anti-mouse IgG) were from Invitrogen Corp (Carlsbad, CA). Human AE5/keratin (K) 3 mAb and goat antibodies against human Tcf4 and survivin were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rabbit antibodies against β-catenin and β-actin were from Cell Signaling Technology (Beverly, MA). Human p63 (4A4), integrin β1 and EGFR mAbs were from Lab Vision (Fremont, CA). HRP conjugated secondary antibodies (goat anti-mouse, goat anti-rabbit and rabbit anti-goat for western blot were from Thermo Scientific (Fremont, CA). Ready gels, Precision Plus Protein Unstained Standards and Precision Protein Streptactin-AP Conjugate came from Bio-Rad (Hercules, CA). The BCA protein assay kit was from Pierce Chemical (Rockford, IL). RNeasy® Mini kit, siRNA-F and HiperFect transfection reagent were from Qiagen (Valencia, CA). Ready-To-Go You-Prime First-Strand Beads were from GE Healthcare (Piscataway, NJ). TaqMan® Gene Expression Assay, real-time PCR Master Mix, and Silencer Select® pre-designed small interfering RNA (siRNA) were from Applied Biosystems (Foster City, CA). WST-1 proliferative assay kit was from Roche Molecular Biochemicals (Mannheim, Germany). Human insulin, transferrin, sodium selenite, hydrocortisone, epidermal growth factor (EGF), cholera toxin A subunit, propidium iodide (PI) and all other reagents came from Sigma–Aldrich (St. Louis, MO).

2.2. Human corneal epithelial cell (HCEC) cultures in different media

Primary HCECs were cultured from donors' limbal tissue explants using a previously described protocol (Kim et al., 2004; de Paiva et al., 2006a). In brief, the limbal ring is cut into 12–16 pieces with similar size of approximately 2 mm × 2 mm each. Two pieces with the epithelium side up were directly put into a well of 6-well plate or one piece per chamber in 8-chamber slides. Low calcium and serum free progenitor cell culture media, CnT-20, CnT-50, KSFM and D-KSFM, and high calcium serum-containing supplemental hormonal epithelial medium (SHEM) were used for cultures at 37 °C under 5% CO_2 and 95% humidity. The media were changed every 2–3 days.

Corneal epithelial cell growth was carefully observed and photographed through a Nikon TE200 inverted phase microscope with a Nikon DXM1200 digital camera. Only the epithelial cultures without visible fibroblast contamination were used for this study. When grown to 90% confluence, the cultures were photographed and trypsinized with 0.25%trypsin/0.03%EDTA; and the cells were seeded into a new plate at a density of 2×10^4 cells/cm^2 for serial passages.

2.3. Colony forming efficiency (CFE) and growth capacity

To evaluate proliferative capacity of corneal epithelial cells in different culture media, the CFE was assessed in cultures in CnT-20 or SHEM using a previous method (Rheinwald and Green, 1975; Li et al., 2005; de Paiva et al., 2005) with modification. Primary human corneal epithelial cells were seeded in triplicate at 500 cells/cm^2 into six-well culture plates without 3T3 fibroblasts or any other cells as a feeder layer. Colonies with more than eight viable cells were counted manually under an inverted phase microscopy at days 6 and 8. Experiment was repeated at least three times. The CFE in SHEM or CnT-20 was calculated as a percentage of the number of colonies generated by the number of epithelial cells plated in a well. The growth capacity was evaluated on day 14 when cultured cells were stained with 1% rhodamine.

2.4. RNA interference

To explore the functional role of Tcf4 signaling, RNA interference experiments were performed using our previous method (Chen et al., 2006a; Ma et al., 2010) with modification. In brief, primary HCECs at a density of 5×10^4 cells/cm^2 were transfected with annealed double-stranded siRNA specific for Tcf4 (siRNA-Tcf4, ID. s13863 containing a pool of 3 target-specific 20–25 nt siRNAs) at different concentrations (10 nM, 25 nM and 50 nM), with a non-coding sequence siRNA-fluorescein (siRNA-F, UUCUCCGAACGUGUCACGU) as a negative control (also serve as visible monitor for transfection efficiency) using fast-forward transfection method with HiperFect reagent according to a manufacturer's protocol. The transfection efficiency in HCECs with different concentrations of siRNA-F (10, 25 and 50 nM) after 24 h was 81.4 ± 3.5%, 83.5 ± 4.1% and 87.2 ± 4.3%, respectively, as analyzed by flow cytometry. After incubation for additional 24–72 h, the cells were collected for RNA extraction or protein lysate preparation for further evaluation. The cell viability after transfection for 4 days was